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RESPONSE SPECTRUM OF PENTACHLOROBENZENE AND FLUORANTHENE FOR *CHIRONOMUS TENTANS* AND *HYALELLA AZTECA*

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Abstract—The whole-body residues of pentachlorobenzene (PCBz) and fluoranthene (FLU) in *Hyalella azteca* and *Chironomus tentans* were determined for a variety of chronic sublethal effects. The endpoints evaluated for *H. azteca* included 28-d growth and survival and 42-d growth, survival, and reproduction. Adverse effects to *C. tentans* also were determined at multiple endpoints including 10-d growth, cumulative pupation and emergence, and reproduction. The lowest-observed-effect residue (LOER) based on whole-body residues associated with growth was consistent between compounds and species tested with concentrations ranging from 0.17 to 0.33 μmol/g. For *H. azteca*, the most sensitive endpoints were growth at 0.23 μmol/g and reproduction at 0.11 μmol/g for PCBz and FLU, respectively. For *C. tentans*, the most sensitive endpoints were emergence, development and reproduction at 0.02 μmol/g, and development and reproduction at 0.15 μmol/g for PCBz and FLU, respectively. Compared to residues associated with acute lethality, the most sensitive sublethal endpoints were approximately 4 and 60 times lower for PCBz and FLU, respectively. The relative consistency of the sublethal endpoints suggests that body residues can be a valuable tool to evaluate bioaccumulation data as part of a risk assessment to predict adverse effects to biota.

Keywords—Aquatic invertebrates Fluoranthene Pentachlorobenzene Sublethal body residue

INTRODUCTION

Traditional hazard assessments have relied on the use of environmental media concentrations to assess risk to biota. However, the effectiveness of this approach has been questioned due to the complicating factors of bioavailability and multiple exposure routes, which may alter the interpretation of the results [1]. As such, the use of body residues as a surrogate for the concentration of a chemical at the target site rather than environmental concentrations has been proposed as the dose metric for evaluating toxicity [1,2]. Using the body residue as the surrogate should reflect the toxic potential of the contaminant at the target site and the genetic variation among organisms, thus providing a clearer estimation of potential effects [3,4].

The body residue approach, linking internal residues to toxicological effects, has focused primarily on acute responses (i.e., mortality), with acute toxicity of fish for narcotic contaminants reported to range from 2 to 8 μmol/g [1,2,5,6]. Narcotic chemicals, for the purposes of the present study, are defined as anesthetics and are assumed to elicit their toxic effects by disrupting the lipid bilayer, resulting in loss of selective permeability and ultimately death. In addition to fish, this approach has been applied to other organisms including a variety of invertebrate taxa [7–9]. Aquatic invertebrates seem to be somewhat more sensitive to narcotic compounds than fish, where the acute toxicity of many species have been shown to be <2.0 μmol/g [4,10–12].

However, the absence of mortality does not necessarily mean the absence of deleterious effects. Exposure to narcotic chemicals also may affect a variety of sublethal processes including growth, reproductive capacity, and developmental time [3,4,13]. Therefore, it is important to consider sublethal as well as lethal effects, because contaminant concentrations in aquatic environments required to produce sublethal effects occur more frequently than do concentrations required for lethal effects. Finally, body residues associated with adverse sublethal effects may be useful for determining the likelihood of effect in organisms collected from the field. However, there are currently limited data available for these types of effects on a body residue basis for most species.

The objectives of the present study were to establish a response spectrum based on internal body residues for pentachlorobenzene (PCBz) and fluoranthene (FLU), ranging from lethality to a series of sublethal endpoints, for *Hyalella azteca* and *Chironomus tentans*.

MATERIALS AND METHODS

Organisms

The amphipod, *H. azteca* (juvenile), and the midge, *C. tentans* (second instar), were chosen because they are recommended by the U.S. Environmental Protection Agency (U.S. EPA) for sediment toxicity testing [14] and because of their ecological importance and geographical distribution. All organisms are currently cultured at Southern Illinois University Carbondale in accordance with U.S. EPA methods [14] and approved institutional animal care and use committee protocols.

Chemicals

The (^{14}C) PCBz (specific activity, 10 mCi/mmol) and (^{3}H) FLU (specific activity, 100 mCi/mmol) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Unlabeled compounds

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were obtained from Chemservice (West Chester, PA, USA). Labeled compounds were tested for purity using high-pressure liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) followed by liquid scintillation counting (LSC) using a Packard TriCarb 2900TR Liquid Scintillation Analyzer (Packard Instrument, Meriden, CT, USA). Purities of the radiolabeled PCBz and FLU were determined to be >98%. The purity of the unlabeled compounds were >98% as indicated by the manufacturer. Stock solutions were prepared by adding known quantities of labeled compound to known amounts of unlabeled compound using acetone as a carrier. The specific activities were recalculated by adjusting for the isotopic dilution.

Sublethal bioassays

All experiments employed an aqueous exposure route. Moderately hard exposure water (MHW) was prepared by adding the necessary salts to deionized water and then allowing the water to mix overnight to ensure the required water quality [15]. The spiking procedure consisted of adding the predetermined amount of contaminant(s) (labeled and unlabeled) using acetone as a carrier solvent to a bulk aliquot of water. The volume of carrier (<100 μl/L) was equal across all exposures.

The sublethal endpoints monitored for *H. azteca* included 28-d survival and growth and 42-d survival, growth, and reproduction. Ten organisms (~7 d old) per beaker were exposed to a series of five concentrations in 300-ml beakers using 12 replicates. A layer of sterile sand (20 g, Fisher Scientific, Hampton, NH, USA) was added to each beaker as substrate. Organisms were exposed using a static renewal system, in which the exposure water was renewed (2 volumes/d) and organisms fed daily (6 mg Tetramin®, Tetra, Blacksburg, VA, USA). Following the 28-d exposure, growth and survival were determined from four replicate beakers, where growth was estimated as organism length (±0.1 mm). Organisms were preserved in a 10% formalin solution until length measurements could be taken. Total length was calculated from the base of the first antennae to the tip of the third uropod from digital photographs and the digitizing software Image J (Image J 1.32j, National Institutes of Health, Bethesda, MD, USA). Three additional replicates were removed at 28 d, five organisms were removed from each replicate, rinsed, weighed, and body residues determined by LSC. The remaining organisms from each replicate were frozen for analysis of biotransformation as described below.

Following the U.S. EPA sediment testing protocols [14], *H. azteca* exposures were terminated after 28 d and surviving amphipods from the remaining five replicates were transferred to water-only beakers containing control water (MHW). Cumulative reproductive output was monitored for an additional 14 d. On days 35 and 42, the adults from each replicate were removed and the numbers of young counted. Additionally, at 42 d, survival and growth was determined as previously described. The number of adult females was determined by counting the adult males, which have an enlarged second gnathopod, and subtracting them from the total to give the number of adult females [14]. The number of females was used to determine reproductive effects by comparing the number of offspring per female. Because the reproductive assessments were conducted in control water following the 28-d exposures to PCBz and FLU, the endpoint response was linked to the 28-d residues.

Sublethal tests using *C. tentans* were conducted using growth and development time as endpoints. Growth was examined by exposing 10 organisms (second instar) per replicate to a series of five concentrations in 500-ml beakers using eight replicate beakers. Sterile sand (20 g) was added for substrate to minimize cannibalism. Exposure waters were 90% renewed and the organisms were fed 6 mg Tetramin daily. After a 10-d exposure, organisms from three replicate beakers in each treatment were selected for residue analysis. From these beakers, five organisms were removed, weighed, and body residues determined by LSC. The remaining organisms in each replicate were frozen for analysis of biotransformation as described below. Organisms from the five remaining replicates were removed, rinsed with deionized water, and weighed to the nearest 0.1 mg. Dry weights from each replicate were determined by drying to a constant weight at 90°C.

In separate experiments designed to assess development time, midges were exposed in the same fashion as in the growth experiment; however, the exposure was conducted from second instar to adult. Residues at 20 d were selected as the dose metric for these exposures because this corresponded to the time of first pupation in preliminary experiments. The endpoints evaluated in these exposures included time to pupation, cumulative total pupation, time to emergence, cumulative total emergence, egg production, and sex ratio, which were linked to the 20-d whole-body residues. We defined times to pupation and emergence as the total time required for the larvae to develop to the pupal stage or to emerge as adults, respectively. The cumulative pupation and emergence are the total number of organisms reaching each stage.

Analysis of body residues

Whole body residues were determined by LSC after weighing. Organisms were transferred to scintillation vials containing 10 ml of scintillation cocktail (ScintiSafe 50%, Fisher Scientific, Hampton, NH, USA). Samples were then sonicated for 60 s in pulse mode (Tekmar High-Intensity Ultrasonic Processor, Tekmar Corporation, Salon, OH, USA). Samples were then stored for 24 h prior to counting to aid in the final extraction and reduce chemiluminescence. Sample counts were corrected for background and quench using the external standard ratio method, which uses the counting efficiency of a high activity, high energy external standard to assess quenching of each sample that allows conversion of counts per minute data to disintegrations per minute with the use of a pre-established quench curve.

The above procedure describes the analysis for total equivalent residues within the organism. This measure is appropriate only if the compound in question is resistant to biotransformation or the test species does not possess the enzymes responsible for biotransformation. For compounds that are biotransformed, the total radiolabeled residue may overestimate the bioactive body residues when expressed as total equivalents (parent compound and all biotransformation products). Previous research demonstrated that PCBz was not biotransformed by these test species; therefore, the total equivalent concentration is equal to parent compound concentration [12]. However, FLU is biotransformed by both organisms; therefore, the amount of biotransformation was quantified for this compound. This was performed using methods described in Schuler et al. [16].

Briefly, biotransformation was determined following a differential extraction procedure based on a lipid extraction technique [17], which allowed for the determination of FLU residues based on their affinity for the organic or aqueous frac-

tions. The organic fraction contains parent and phase 1 metabolites (toxic fraction), while the aqueous fraction (nontoxic fraction) contains the hydrophilic phase 2 metabolites. Phase 1 metabolites are included in the toxic fraction because the addition of functional groups (e.g., –OH) does not significantly change the lipid solubility of the molecule. Since the mode of action of nonpolar organics is narcosis, the toxicity of the parent and phase 1 metabolites is expected to be similar at the target site within the organism [18]. Unlike the phase 1 biotransformation, the addition of conjugates in phase 2 biotransformation makes the compound much more hydrophilic, reducing its ability to act as a nonpolar narcotic. All body residue interpretations were based on the toxic equivalent fraction that allows for better interpretation of the differences in residue effects data.

Statistics

Survival, pupation, emergence, and reproduction data from the sublethal exposures were compared by ANOVA (one-way analysis of variance) and Dunnett's multiple comparison test [19] were used to determine the no-observable-effect residue (NOER) and the lowest-observable-effect residue (LOER). Prior to these analyses, the data were tested for normality and homogeneity of variance. Where necessary, the data were either arc-sine or log transformed to meet the assumption of the statistical technique. A p value of 0.05 was considered significant for all analyses. A Kolmogorov–Smirnov test with Bonferroni adjustment was selected to determine significance among the development endpoints (cumulative pupation and cumulative emergence). Pair-wise comparisons were made between control and treatment groups only.

A portion of this paper identifies a response spectrum based on body residues ranging from lethality to the various sublethal endpoints described above. The lethal residue data used in the response spectrum were obtained from a previous study [12].

RESULTS

42-d H. azteca *partial–life cycle tests*

Toxic equivalent body residues in *H. azteca* were determined for PCBz and FLU after 28-d exposures and linked to a variety of endpoints including survival, growth, and reproduction following U.S. EPA protocols [14]. For the 42-d endpoints, the 28-d metric was selected because it corresponded to the end of the exposure phase. In addition, if daily renewals of the treatment concentrations had continued during the reproductive period (28–42 d), the reproduction results might have been misleading due to the loss of neonates during water exchange caused by their small size as well as potential loss of neonates from mortality.

Survival of *H. azteca* following the 28-d exposure to PCBz ranged from 73 to 86% with residues up to 0.52 μmol/g and was not significantly different from controls. In the FLU exposures, survival ranged from 47 to 88% and significant mortality was noted at 0.49 μmol/g (Fig. 1). Following the 28-d exposure and the transfer to uncontaminated water, growth and survival were again assessed after a 14-d recovery period. The overall 42-d survival of *H. azteca* for both chemicals decreased slightly, but not significantly compared to the 28-d survival. Since the objective of the present work was to identify residues associated with sublethal effects, the organisms at 0.49 μmol/g FLU residue were not evaluated for the 42-d sublethal endpoints due to significant chronic mortality, and thus, a potential for density dependent results.

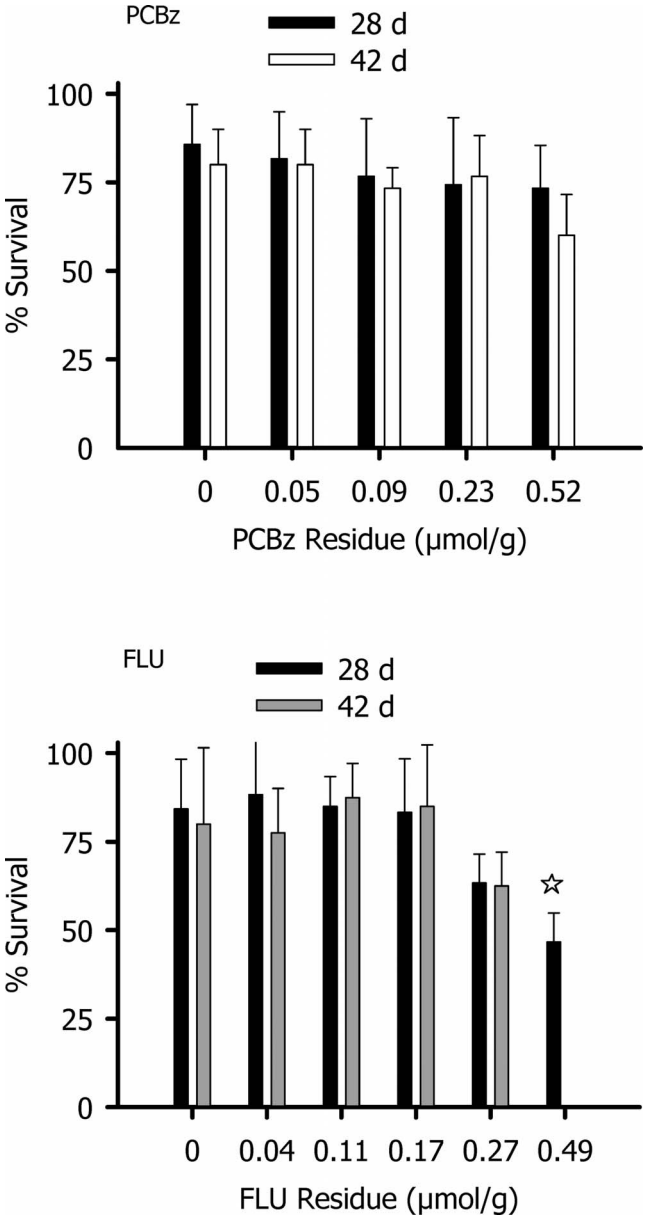


Fig. 1. Survival of *Hyalella azteca* following a 28-d exposure to pentachlorobenzene (PCBz) and fluoranthene (FLU). The 42-d survival represents organisms that were exposed for 28 d and transferred to uncontaminated water for 14 d. Stars indicate statistically significant differences compared to control organisms ($p = 0.05$).

Significant decreases in growth (as measured by total length) of *H. azteca* were found with increasing body residues following 28-d exposures for both PCBz and FLU (Table 1). Additionally, growth of *H. azteca* was compared by sex; males were generally larger than females and the sex-specific growth showed similar trends compared to the combined growth (male and female). Some exceptions were noted; for instance, males were not significantly different from the controls at any of the body residues attained in the PCBz exposure, while the growth of males was slightly more affected in the FLU exposures compared to the controls. Data in the literature are absent with respect to sex-specific accumulation; unfortunately, we did not determine sex-specific residues, so potential accumulation differences are unknown.

Reproduction (number offspring per female) was monitored for 14 d following the 28-d exposures. No significant differ-

Table 1. Mean length of *Hyalella azteca* (mm ± standard deviation) following a 28 d exposure to pentachlorobenzene (PCBz) and fluoranthene (FLU). The 42 d study was composed of a 28 d exposure to a treatment followed by 14 d exposure to control water[a]

Compound	Body residue (μmol/g)	28-d exposure			42-d exposure		
		Combined	Male	Female	Combined	Male	Female
PCBz							
	0	4.53 ± 0.25	4.81 ± 0.31	4.36 ± 0.20	4.47 ± 0.15	4.60 ± 0.07	4.40 ± 0.21
	0.05	4.33 ± 0.33	4.31 ± 0.42	4.27 ± 0.33	4.62 ± 0.23	4.93 ± 0.26	4.34 ± 0.18
	0.09	4.56 ± 0.24	4.70 ± 0.22	4.29 ± 0.19	4.14 ± 0.06	4.63 ± 0.44	3.87 ± 0.16
	0.23	3.75 ± 0.19*	4.22 ± 0.52	3.56 ± 0.25*	4.09 ± 0.13	4.38 ± 0.20	3.85 ± 0.05
	0.52	3.65 ± 0.29*	4.01 ± 0.06*	3.45 ± 0.16*	4.47 ± 0.15	4.60 ± 0.07	4.40 ± 0.21
FLU							
	0	4.68 ± 0.39	5.04 ± 0.30	4.41 ± 0.07	4.31 ± 0.05	4.32 ± 0.15	4.31 ± 0.09
	0.04	4.28 ± 0.19	4.26 ± 0.21	4.29 ± 0.21	4.26 ± 0.07	4.66 ± 0.21	3.94 ± 0.18
	0.11	4.12 ± 0.17	4.14 ± 0.17*	4.03 ± 0.19	4.13 ± 0.12	4.49 ± 0.10	3.88 ± 0.15*
	0.17	3.89 ± 0.18*	4.30 ± 0.10*	3.71 ± 0.28*	3.94 ± 0.14*	4.28 ± 0.26	3.84 ± 0.18*
	0.27	3.56 ± 0.15*	3.80 ± 0.14*	3.50 ± 0.18*	3.41 ± 0.30*	3.90 ± 0.30*	3.57 ± 0.11*
	0.49	3.42 ± 0.10*	3.36 ± 0.10*	3.25 ± 0.17*	NA[b]	NA	NA

[a] Asterisks indicate statistically significant differences compared to control organisms ($p = 0.05$).
[b] NA = not available.

ences in reproduction were detected for the PCBz exposed amphipods up to 0.52 μmol/g compared to the controls (Table 2). For FLU, reproduction decreased with increasing 28-d body residue. The number of offspring/female with residues of 0.11 μmol/g was significantly reduced (37%).

Chironomus tentans *partial–life cycle tests*

The mean dry weight of *Chironomus tentans* following a 10-d exposure to PCBz and FLU generally decreased with increasing body residues (Table 3). In the PCBz exposure, mean midge weight ranged from 2.2 ± 0.1 mg dry weight in the controls to 1.0 ± 0.3 mg dry weight at the highest residue level. Similar results were found for the FLU exposures, where the mean midge weight ranged from 2.6 ± 0.3 mg dry weight in the controls to 1.3 ± 0.4 mg dry weight at the highest treatment level. The mean weight compared to control midges was significantly reduced at 0.33 μmol/g and 0.21 μmol/g for PCBz and FLU, respectively.

Developmental times were assessed using pupation and emergence as endpoints. Midge larvae were sampled at 20 d for residue analysis, which corresponds to the approximate time of first pupation. Development time was consistently longer for midges exposed to PCBz and FLU compared to the control organisms. The delay was significant in all PCBz treatments for pupation and emergence compared to the controls. For example, following a 60-d exposure, the cumulative pupation in the controls was 90 ± 14% (±standard deviation [SD]), whereas the pupation ranged from 70 ± 14% (±SD) at a body residue of 0.02 μmol/g to 45 ± 20% (±SD) at 0.40 μmol/g (Fig. 2). The time required for 50% of the midges to pupate in the control was 28.5 d compared to the 49.4 d at a PCBz body residue of 0.02 μmol/g, the lowest treatment concentration. Although the organisms were exposed up to 60-d, the 20-d endpoint was selected as the dose metric because residues in the organisms were at steady state and the kinetics are sufficiently fast that growth dilution was not expected to be a factor [16]. Additionally, from a practical standpoint, the odds of being able to collect enough pupae in the field for chemical analysis are unlikely; therefore, the residues in the larvae corresponding to the time at or near the first pupation were selected as the metric for the pupation and emergence endpoints.

In the FLU exposures, pupal development was not as impacted as in the PCBz exposures. Cumulative pupation of the controls was consistently higher than the treatments; however, a statistically significant difference was determined only at a body residue of 0.26 μmol/g. The cumulative pupation following 38-d exposure ranged from 80 ± 20% in controls to 52 ± 10% at 0.26 μmol/g. The time required for 50% of the midges to pupate in the control was 30.3 d compared to the 36.8 d at 0.26 μmol/g. There was no difference in pupation between PCBz and FLU controls.

Similar to pupation, the total cumulative emergence was significantly affected in all PCBz treatments (Fig. 3). Since

Table 2. Reproductive output of *Hyalella azteca* following a 28 d exposure to pentachlorobenzene (PCBz) and fluoranthene (FLU)[a]

PCBz		FLU	
Body residue (μmol/g)	Offspring/female	Body residue (μmol/g)	Offspring/female
0.00	7.15 ± 4.46	0.00	4.40 ± 2.16
0.05	5.64 ± 2.33	0.04	4.38 ± 1.14
0.09	4.94 ± 1.25	0.11	1.64 ± 1.00*
0.23	5.03 ± 2.78	0.17	1.05 ± 0.10*
0.52	5.63 ± 1.85	0.27	0.96 ± 0.52*
		0.49	NA[b]

[a] Asterisks indicate statistically significant differences compared to control organisms ($p = 0.05$).
[b] NA = not available.

Table 3. Mean dry mass of *Chironomus tentans* (mg ± standard deviation) following a 10 d exposure to pentachlorobenzene (PCBz) and fluoranthene (FLU)[a]

PCBz		FLU	
Body residue (μmol/g)	Growth (mg dry wt)	Body residue (μmol/g)	Growth (mg dry wt)
0.0	2.2 ± 0.1	0.0	2.6 ± 0.3
0.02	2.1 ± 0.1	0.01	2.3 ± 0.2
0.09	2.2 ± 0.2	0.02	2.5 ± 0.3
0.19	1.8 ± 0.2	0.09	1.9 ± 0.1
0.33	1.6 ± 0.3*	0.14	2.1 ± 0.1
0.51	1.0 ± 0.3*	0.21	1.3 ± 0.4*

[a] Asterisks indicate statistically significant differences compared to control organisms ($p=0.05$).

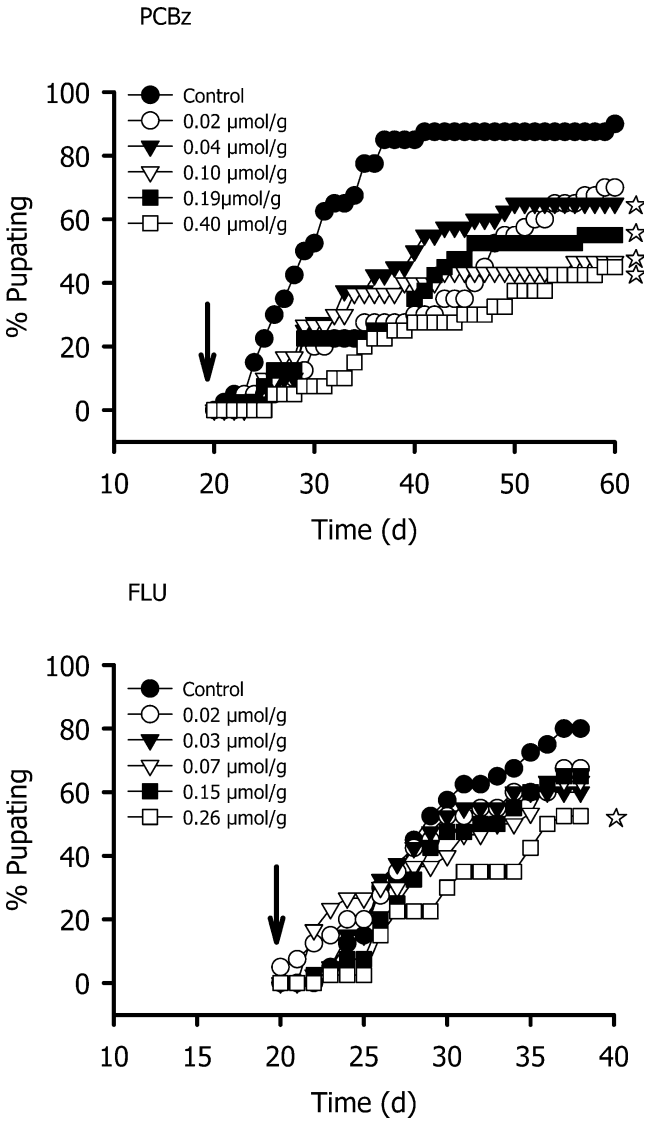


Fig. 2. Cumulative pupation with time for *Chironomus tentans* exposed to pentachlorobenzene (PCBz) and fluoranthene (FLU). Arrow indicates time at which body residues were collected. Stars indicate statistically significant differences in pupation compared to control organisms ($p = 0.05$).

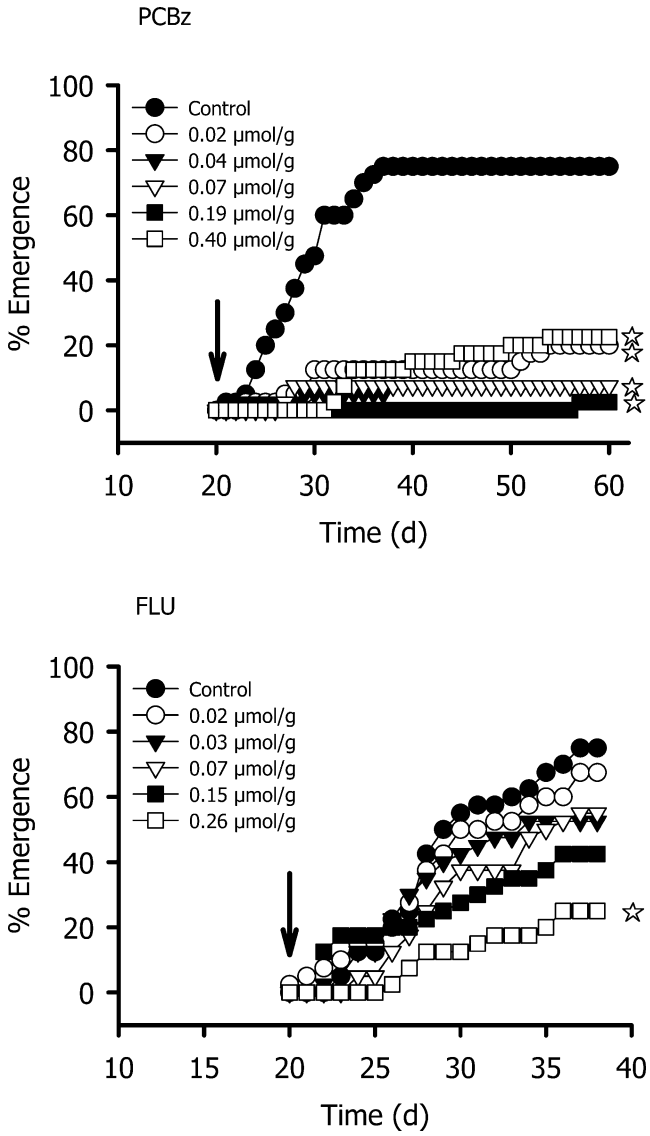


Fig. 3. Cumulative emergence with time for *Chironomus tentans* exposed to pentachlorobenzene (PCBz) and fluoranthene (FLU). Arrow indicates time at which body residues were collected. Stars indicate statistically significant differences in emergence compared to control organisms ($p = 0.05$).

emergence is correlated with pupation, the emergence data were scaled to the number of pupae for each treatment prior to statistical analysis. For example, approximately 90% of the controls pupated; however, 75% of the pupae emerged into adults. This resulted in 84% net emergence in the controls. The net emergence for the PCBz exposed midges was 17, 11, 16, 5, and 49% for 0.02, 0.04, 0.07, 0.19, and 0.40 µmol/g, respectively. The apparent lack of dose–response for the net emergence data was likely due to the overall lack of emergence and biological variation.

For FLU exposures, emergence as with pupation followed the typical dose–response relationship with decreased emergence with increasing residues. Using the statistical procedure described above, significant differences in emergence were detected at 0.26 µmol/g (Fig. 3). The net emergence for the controls was 88% compared to only 50% at 0.26 µmol/g. The remaining FLU treatments ranged from 85 to 100% net emergence. The relatively high percentage of net emergence in the FLU exposures allowed for sex-specific comparisons of the time to emergence (Fig. 4). In all cases, the time required for first emergence for females was longer than first emergence in males. The mean difference between first emergences was approximately 6 ± 1.5 d across all treatments except the highest exposure where female emergence was significantly reduced.

Reproduction was determined as the number of eggs per female (Table 4). For FLU, reproduction was significantly lower at 0.15 µmol/g compared to the control. For PCBz, statistical significance was not calculated due to low numbers of emerging adults; however, there were no differences in reproduction between PCBz and FLU controls.

Survival of the midges was determined following the end of the time course for each compound (60 d PCBz, 38 d FLU). Survival was determined as the sum of the number of larvae that pupated and the number of larvae that remained following the termination of the test (Fig. 5). Survival in the PCBz exposures was significantly affected at residues of 0.10

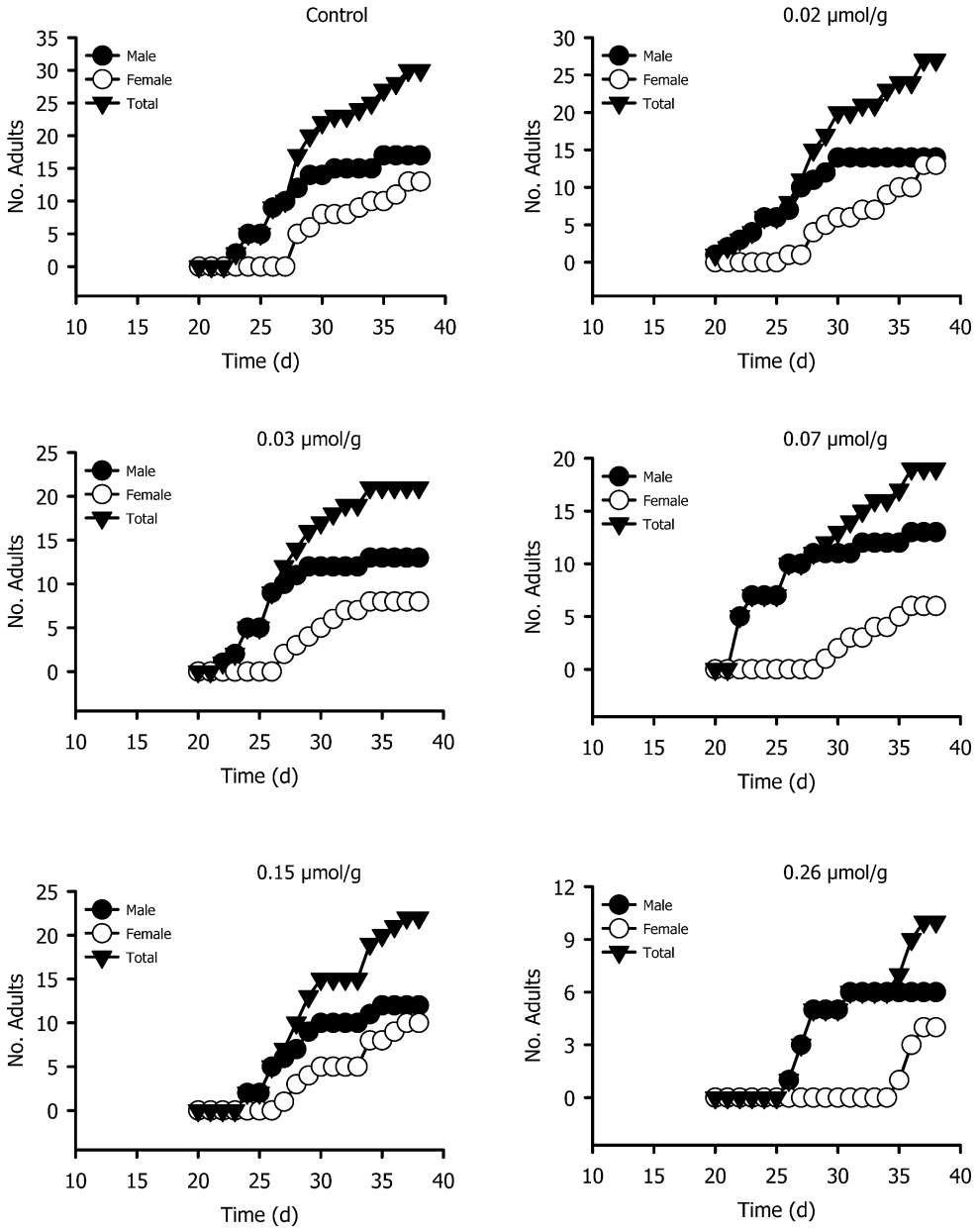


Fig. 4. Sex-specific emergence with time for *Chironomus tentans* exposed to fluoranthene.

Table 4. Reproductive output of *Chironomus tentans* following exposure to pentachlorobenzene (PCBz) and fluoranthene (FLU)[a]

PCBz			FLU		
Body residue (µmol/g)	Egg/female	*n*	Body residue (µmol/g)	Egg/female	*n*
0.0	1,025 ± 254	9	0.0	1,347 ± 301	7
0.02	625 ± 35	2	0.02	983 ± 157	8
0.04	728	1	0.03	1,104 ± 255	8
0.10	1,300	1	0.07	952 ± 28	2
0.19	NA[b]	—	0.15	844 ± 469*	8
0.40	457 ± 389	3	0.26	NA ± NA	—

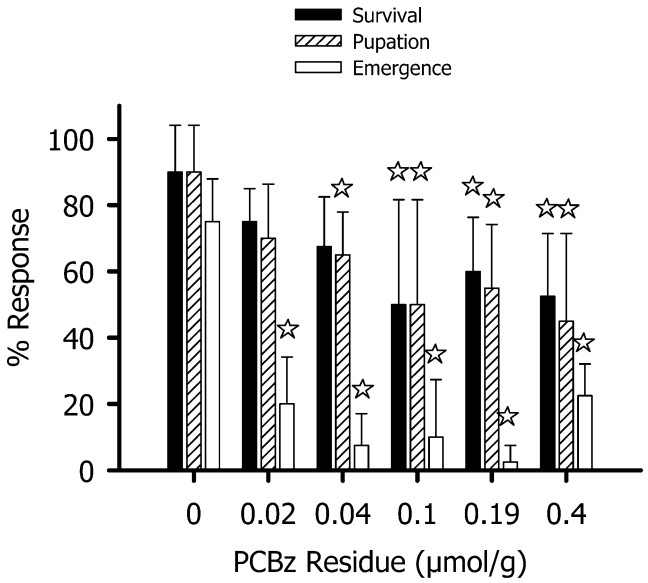
[a] Asterisks indicate statistically significant differences compared to control organisms ($p = 0.05$).
[b] NA = not available.

µmol/g and greater. No differences in survival were detected for FLU at residues of 0.26 µmol/g.

Response spectrum

The overall goal of the present study was to develop a response spectrum of adverse biological effects for *H. azteca* and *C. tentans* to PCBz and FLU. For *H. azteca*, growth was the most sensitive endpoint for PCBz, where the NOER and the LOER were 0.09 and 0.23 µmol/g, respectively (Fig. 6). Reproduction and survival were not significantly affected at residues up to 0.52 µmol/g. Previous results for *H. azteca* have determined the 28-d lethal residue causing 50% mortality (LR50) for PCBz to be 0.71 µmol/g [12]. This supports the lack of mortality in the current work. From these data, the maximum allowable toxicant residue (MATR), determined from the mean of the NOER and LOER, for all endpoints examined was 0.16 µmol/g.

In the FLU exposures, reproduction was the most sensitive



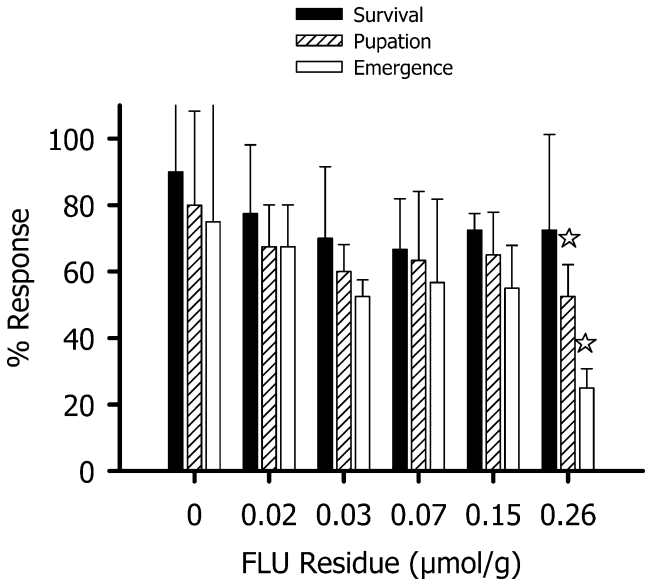
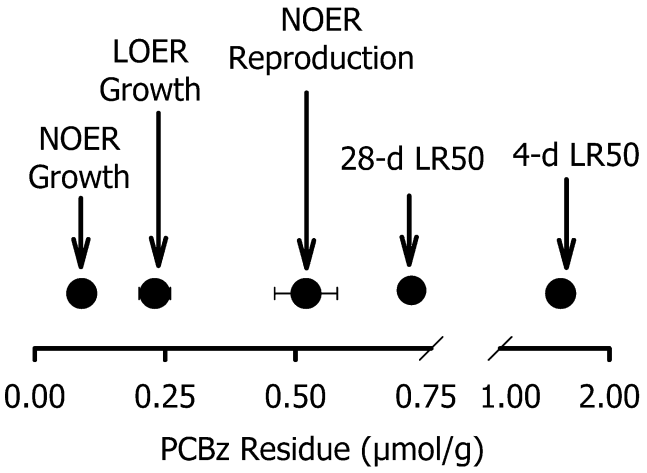


Fig. 5. Response summary for *Chironomus tentans* following exposure to pentachlorobenzene (PCBz) and fluoranthene (FLU). Stars indicate statistically significant differences compared to control organisms ($p = 0.05$).



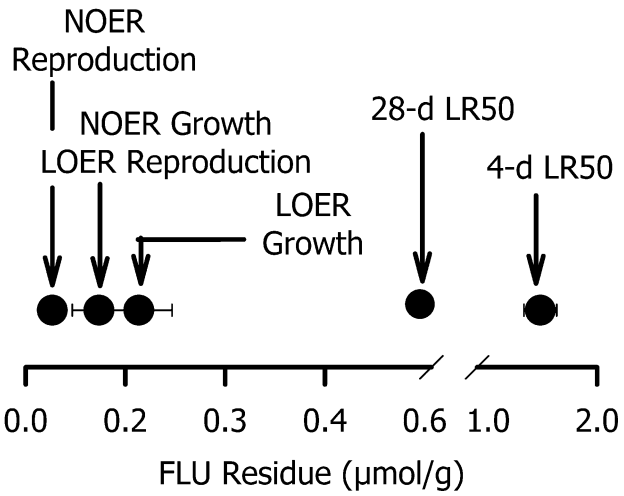


Fig. 6. Response spectrum of biological effects ranging from mortality to sublethal effects (e.g., growth and reproductive effects) for *Hyalella azteca* exposed to pentachlorobenzene (PCBz) and fluoranthene (FLU). All residues are expressed as toxic equivalents. LR50 = lethal residue; NOER = no-observed-effects residue; LOER = lowest-observed-effects residue.

endpoint, where the NOER and LOER values were 0.04 and 0.11 µmol/g, respectively (Fig. 6). The NOER and LOER values for growth were 0.11 and 0.17 µmol/g, respectively. Survival was significantly affected at the highest residue levels tested and the NOER and LOER values were 0.27 and 0.49 µmol/g. The survival data follows previous work where the reported 28-d LR50 value was 0.56 µmol/g [12]. From this data, the MATR of FLU for *H. azteca* is 0.08 µmol/g.

For *C. tentans* exposed to PCBz, larval development, emergence, and reproduction were the most sensitive endpoints (Fig. 7). The LOER value for these endpoints was 0.02 µmol/g. The NOER and LOER values for growth were 0.19 and 0.33 µmol/g, respectively. The NOER and LOER values for survival were 0.04 and 0.10 µmol/g, respectively. Previous results have determined the 10-d LR50 as 0.81 µmol/g [12]. The MATR for PCBz was 0.01 µmol/g.

In the FLU exposures, developmental delays and reproduction were the most sensitive endpoints (Fig. 7). The NOER and LOER for reproduction and development were 0.07 and 0.15 µmol/g. The NOER and LOER values for pupation and emergence were 0.15 and 0.26 µmol/g, respectively. Growth was similarly affected with NOER and LOER values of 0.14 and 0.21 µmol/g. The sublethal residues we determined were similar to the previously identified 10-d LR50 value of 0.15 µmol/g. The MATR for FLU was 0.11 µmol/g.

DISCUSSION

The data generated from this research attempted to improve hazard assessments by linking body residues to toxicological responses in *H. azteca* and *C. tentans*. In the environment, these aquatic organisms are more likely to be exposed to contaminant concentrations that are lower than those required for lethality; however, the absence of lethality does not necessarily mean the absence of adverse effects. A number of sublethal

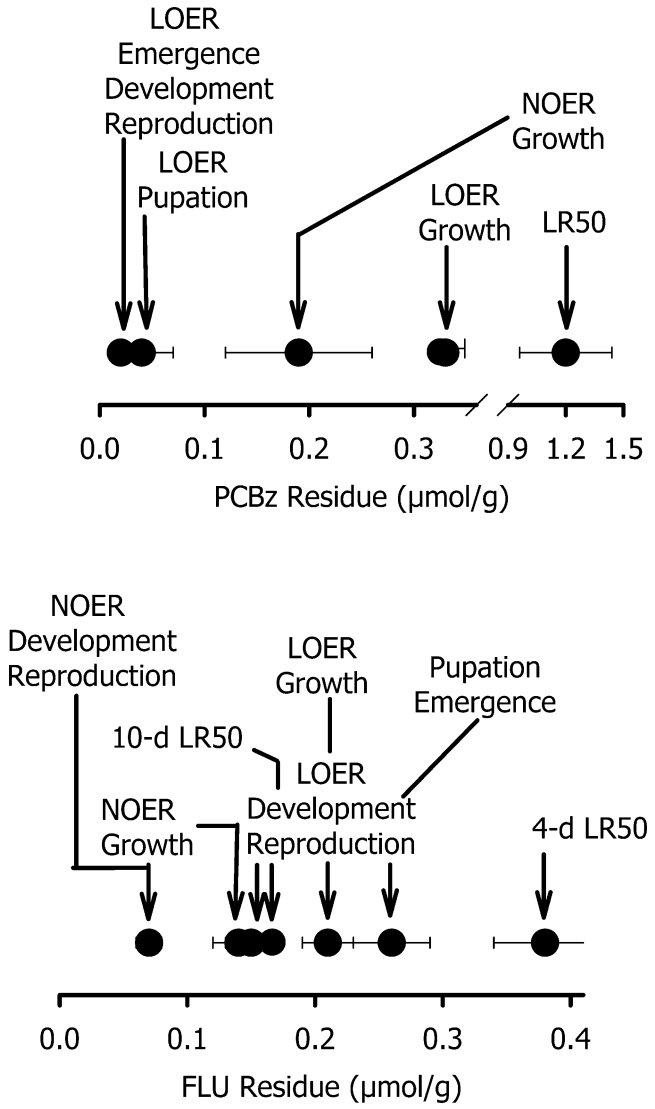


Fig. 7. Response spectrum of biological effects ranging from mortality to sublethal effects (e.g., growth and reproductive effects) for *Chironomus tentans* exposed to pentachlorobenzene (PCBz) and fluoranthene (FLU). All residues are expressed as toxic equivalents. LR50 = lethal residue; NOER = no-observed-effects residue; LOER = lowest-observed-effects residue.

effects in response to contaminants have been identified for a variety of invertebrate species including behavioral changes [20,21], physiological changes [22,23], and reductions in biomass and reproduction [8,11,24]. Generally, these effects are evident at body residues below those required for lethality. Further, environmental exposures may at times be intermittent or pulsed exposures as opposed to the more continuous exposures used in the present study. The relatively continuous exposures should yield a better estimate of the lower limit for body residue required for chronic responses because there are no recovery times available to the organism. Therefore, these sublethal body residues and the resulting response spectrum are expected to prove useful in the application of the body residue approach since such estimates are generally missing from the literature.

As a means to gauge the relative sensitivity of the sublethal endpoints to be included in a residue-response spectrum, residues associated with acute lethality were identified from a previous study [12]. The 4-d acute lethal residue causing 50% mortality (LR50) for *H. azteca* was approximately 1.5 and 1.9 µmol/g for PCBz and FLU, respectively. The 4-d acute LR50 values for *C. tentans* corresponded to 1.2 and 0.4 µmol/g for PCBz and FLU, respectively [12].

Adverse sublethal effects were expected to present themselves prior to lethality and the data from the present study indicate that a number of sublethal effects were more sensitive endpoints than acute lethality and can be predicted using the body residue approach. The most sensitive sublethal effects for the species tested ranged from approximately 4 to 60 times lower than residues associated with the acute LR50 values above. For instance, the most sensitive sublethal endpoints for *H. azteca* were growth at 0.23 µmol/g and reproduction at 0.11 µmol/g for PCBz and FLU, respectively. For *C. tentans*, the most sensitive endpoints, emergence and development, correspond to 0.02 and 0.15 µmol/g for PCBz and FLU, respectively. In addition to being more sensitive than acute lethality [12], residues associated with sublethal effects were generally lower than those identified for chronic mortality (see Figs. 6 and 7). Therefore, whole-body residues for individual compounds can be used to predict sublethal effects that are likely to have population level impacts.

These findings are supported in the literature, where several studies have reported body residues associated with sublethal impairments to be at much lower levels than residues implicated in lethality. For example, Lotufo [8] determined 50% reductions in reproduction and feeding for copepods exposed to FLU at body residues ranging from 0.2 to 0.7 µmol/g, compared to the 10-d lethal body residue of 2.7 µmol/g. The FLU residues required to produce these sublethal effects in copepods are similar to residues for other narcotic chemicals, including the 0.34 to 0.56 µmol/g for several polychlorinated biphenyls (PCBs) determined by Fisher et al. [3] that affect growth and reproduction in the oligochaete, *Lumbriculus variegatus*, and PCB residues of 0.015 to 0.44 µmol/g determined by Hwang et al. [4,11] for a variety of life cycle effects in *Chironomus riparius*. In addition to being in the range of sublethal effects shown for other invertebrates, the residues causing sublethal effects determined in the present study are similar to those determined for fish. Reduced growth in juvenile *Pimephales promelas* was demonstrated for organisms with residues of 0.02 µmol/g and 0.66 µmol/g for FLU and PCBz, respectively [25]. The current data together with these results, suggest that sublethal residues for individual narcotic contaminants, while lower than for lethality, are relatively similar for many sublethal endpoints. Moreover, assuming that the effects are determined by a narcotic mechanism of action, the number of molecules in the membrane; it would stand to reason that the actual contaminant is unimportant as long as the chemical acts by nonpolar narcosis. Given this, it seems reasonable that in a field situation where toxicity is expected to be due to a myriad of contaminants at relatively low concentrations, the overall toxic effect may be predicted from the molar sum of all nonpolar contaminants.

Not all sublethal endpoints were equally sensitive with respect to body residue or followed a pattern with more biologically complex endpoints such as reproduction exhibiting the greatest sensitivity. For instance, because *H. azteca* is known to exhibit size-specific fecundity [14,26], residues required to reduce reproduction were expected to follow growth in terms of endpoint sensitivity. As expected for FLU, reductions in reproduction paralleled reductions in growth with an LOER value of 0.11 µmol/g. However, for PCBz, no significant re-

ductions in reproduction were found at residues up to 0.52 μmol/g approaching concentrations that produce 50% lethality 0.71 μmol/g [12]. Thus, reduction in reproduction did not parallel growth, which was much more sensitive with an LOER of 0.09 μmol/g. The sensitivity of *H. azteca* to PCBz for growth was different in the present study compared to the sensitivity reported in the literature for PCBz and other chlorinated benzenes. For example, the estimated PCBz concentration required to cause a 50% reduction in growth was 0.87 μmol/g, which is similar to or above the reported LR50 values [9,16]. For the structurally similar compound, hexachlorobenzene had no effect on growth or reproduction for *H. azteca* at concentrations of 0.60 μmol/g [24]. The reason for the observed difference in the two PCBz studies could lie in the method of analysis of the data and variability in organism response.

Although reproduction did not statistically decline with PCBz exposures the reproduction did tend to be lower. The lack of significance may result from high variability in the data resulting in low power for the test. The coefficient of variation for reproduction within PCBz treatments ranged from 25 to 55% and the control was 62%. These values were much greater than the expected 20% CV (coefficient of variation) for reproduction and somewhat greater than the observed variation in the FLU test (10–55%), which affects the statistical power of the test. Thus, significance requires more replicates or larger differences in reproductive output to detect differences [14,27]. Additionally, the failure to find significant results for the reproductive endpoint for PCBz was due to the difference in growth between the controls and the most affected group, which was only approximately 20% at 28 d and 9% at 42 d, suggesting substantial recovery of the amphipods during the 14-d (from 28 to 42 d) reproductive period. Similarly, the reduction was 27% at 28 d for FLU-exposed organisms and 21% at 42 d, suggesting much less recovery that parallels the decline in reproduction. Further, sex-specific growth did not appear to be driving reproduction, because statistical differences in male and female *H. azteca* generally tracked with those determined from the combined analysis, where growth was determined from both sexes (Table 1). The ratio of male to female length was similar among all treatments and matched well with previous data [28], indicating that both sexes were affected equally by the contaminant.

Although amphipod reproduction between experiments exceeded the suggested control average of two offspring per female [14], there was a large amount of variability in reproductive output between control groups (7.15 and 4.40 offspring per female for PCBz and FLU, respectively). The reproduction endpoint was more variable than the other endpoints, where CVs ranged from 5 to 12% in the controls. This variability suggests that reproduction may not be as useful a measure of contaminant effect as survival or growth.

In addition to some variability in endpoint sensitivity for the *H. azteca* exposures, some differences also were noted in the *C. tentans* experiments. The 10-d growth for *C. tentans* was generally the least sensitive sublethal endpoint evaluated, suggesting that using mass as a surrogate for sublethal effects would not be the most protective. This follows data from Hwang et al. [11], where body weight of *C. riparius* determined from pupae was the least sensitive endpoint for several nonpolar contaminants.

Reproduction and developmental times for *C. tentans* followed our expectations that this more biologically complex endpoint would be more sensitive. This result is not surprising considering the complicated life history of *C. tentans* compared to that of many other invertebrate species such as amphipods that undergo direct development. Development and metamorphosis in insects are orchestrated by hormones including juvenile hormone, eclosion hormone, and others. Changes in the homeostasis of one or more of these hormones could result in abnormal growth and development [29]. This was seen at the end of the 60-d PCBz experiment, where the cumulative emergence was significantly reduced compared to cumulative pupation. Cumulative emergence also was reduced compared to pupation in the FLU treatments; however, the extent was not as great. The mechanisms by which these nonpolar compounds delay or prevent pupation and emergence is uncertain because the chemical structures of the two contaminants are not similar to those previously identified compounds having more specific modes of action (e.g., hormone mimics or agonists). However, they may act indirectly by altering hormone levels by interfering with biochemical processes associated with the production, availability, or metabolism of hormones. Although the availability of sublethal effect data is limited, the residues associated with the sublethal effects found here are generally in the range of those expected for narcotic chemicals [3,11,30]. The exact mechanism by which narcosis causes sublethal toxicity is unknown, but has been hypothesized to occur by altering how organisms allocate energy [31]. In a very simplistic view, assimilated energy can be thought of as the currency the organism has to spend on maintenance, growth, and reproduction. In response to a contaminant, the organism has to direct energy to cope with the contaminant stress that would otherwise be directed to growth and reproduction. This was particularly evident in *C. tentans* exposures, where increasing body residues were translated into increased developmental times. The physiology of the midge determines that the metamorphosis from the larval stage to the pupa is achieved only after a critical mass is attained. Therefore, energy used for coping with or repairing damage (increased maintenance costs) caused by the accumulation of contaminants results in slower growth, which translates into longer pupation times. From the data collected, it was not possible to identify the mechanism by which narcotic chemical stress interferes with growth and reproduction; however, several plausible hypotheses have been put forward including: reduced feeding activity [8,21], increased biotransformation rates [31], increased damage repair, reduced protein synthesis [31], and reduced assimilation efficiency [32]. Also, it's possible, if not probable, that multiple processes occurring simultaneously are responsible for the observed effects.

An additional consideration not evaluated in the present study is how the sublethal effects influence the overall survival of a population. In terms of reproduction, the outcome is straightforward; a reduction in offspring will result in the decline or elimination of a population. Increased development time may result in fewer reproductive events during the breeding season due to fewer adults [4] and decreased brood production [33] yielding fewer offspring and possibly a smaller population. Additionally, for populations of organisms in which predation is focused primarily on small individuals, benefits accrue from rapid growth. Delayed growth means that they remain in vulnerable size classes for extended periods of time, leading to possible increased predation [4,26].

Overall, this use of body residues as a dose metric appears to be a promising approach for evaluating risk from bioac-

cumulation data. This will allow direct comparisons of bioaccumulation data of aquatic organisms obtained from routine biomonitoring activities or as part of a hazard assessment to a residue-effects plot that will show potential impacts.

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